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Activities of Daptomycin and Vancomycin Alone and in Combination with Rifampin and Gentamicin against Biofilm-Forming Methicillin-Resistant *Staphylococcus aureus* Isolates in an Experimental Model of Endocarditis[▽]

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The findings of clinical and in vitro research support the theory that infective endocarditis (IE)-causing bacteria form biofilms and that biofilms negatively affect treatment outcomes. The purpose of the present study was to quantify the biofilm formation of methicillin (meticillin)-resistant *Staphylococcus aureus* (MRSA) isolates obtained from patients with IE and to evaluate the in vitro activities of daptomycin and vancomycin alone and in combination with rifampin (rifampicin) or gentamicin while monitoring the isolates for the development of resistance. A high-inoculum, stationary-phase infection model of IE was used to simulate the pharmacokinetics in humans of daptomycin at 6 mg/kg of body weight/day, vancomycin at 1.25 g every 12 h (q12h) alone and in combination with rifampin at 300 mg every 8 h, and gentamicin at 1.3 mg/kg q12h. Two randomly selected clinical MRSA isolates were obtained from patients with IE; both MRSA isolates quantitatively produced biofilms. The time to bactericidal activity in the presence of daptomycin was isolate dependent but was achieved by 24 h for both MRSA isolates. Vancomycin did not achieve bactericidal activity throughout the experiment. At 24, 48, and 72 h, daptomycin-containing regimens had significantly more activity (greater declines in the mean number of CFU/g) than any of the vancomycin-containing regimens ($P = 0.03$). Rifampin and gentamicin antagonized or delayed the bactericidal activity of daptomycin (against MRSA B346846 for rifampin and against both isolates for gentamicin) in the first 24 h. Increases in the daptomycin and vancomycin MICs were not observed. We conclude that in an IE model of biofilm-forming MRSA, daptomycin monotherapy has better in vitro activity than daptomycin in combination with rifampin or gentamicin or any vancomycin-containing regimen studied within the first 24 h. Further investigations are needed to understand the initial delay in bactericidal activity observed when gentamicin or rifampin is combined with daptomycin.

Biofilm-forming *Staphylococcus aureus* isolates are frequently found on prosthetic devices and in deep tissue infections (21, 43), and both prosthetic devices and deep tissue infections serve as common sources for bacteremia. Clinical research supports the theory that infective endocarditis (IE)-causing bacteria form biofilms (14, 19) and that *S. aureus* isolates recovered from the blood of patients with IE tend to produce biofilms at a high inoculum and during the stationary phase of growth (24, 43). These isolates also typically carry accessory gene regulator (*agr*) groups I and II, which regulate the production of autolysins that promote biofilm formation (21, 43, 44). Despite this information, there are limited data about the biofilm-forming capabilities of *S. aureus* isolates that cause IE (SAIE).

Until recently, effective antimicrobial therapy for methicillin (meticillin)-resistant *S. aureus* (MRSA) bacteremia and IE was limited to vancomycin. Although vancomycin has commonly

been used since the 1980s for the treatment of MRSA infections, including endocarditis, several published studies indicate that it has limited efficacy because of bacterial resistance, bacterial tolerance, and poor tissue penetration (8, 22, 36). Patients with SAIE treated with vancomycin alone may still be bacteremic (as indicated by positive blood cultures) after 7 to 10 days of therapy (25, 26). Guidelines for the treatment of SAIE recommend the use of combination therapy with vancomycin plus gentamicin or rifampin (rifampicin) (2, 3, 41). However, these combinations can be problematic because gentamicin increases the risk of nephrotoxicity and rifampin increases the potential for drug interactions via its induction of cytochrome P450 metabolism. In addition, this recommendation is based on limited clinical data.

Daptomycin, a novel lipopeptide antimicrobial agent, received FDA approval in May 2006 for the treatment of bacteremia and right-sided SAIE caused by methicillin-susceptible and -resistant strains (12). The FDA indication is for monotherapy against gram-positive pathogens; however, the guidelines recommend combination therapy for SAIE. Data to support the optimal dose of daptomycin required for it to have activity when it is combined with commonly used synergistic agents such as gentamicin and rifampin are lacking. In addition, limited information about the activity of daptomycin in the pres-

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ence of biofilm-forming *S. aureus* has been published (23, 32, 35, 37).

The purpose of this study was to quantify the biofilm formation of *S. aureus* isolates obtained from patients with SAIE, to assess the in vitro activities of daptomycin and vancomycin alone and in combination with rifampin or gentamicin, and to evaluate the development of resistance in a high-inoculum, stationary-phase bacterial (biofilm) model of IE.

(This work has been presented in part at the 108th General Meeting of the American Society for Microbiology, Boston, MA, 1 to 5 June 2008 [abstr. A-076].)

MATERIALS AND METHODS

Bacterial strains. Clinical MRSA isolates B346846 and B341002 were obtained from the Cubicin bacteremia and IE registration trial (ClinicalTrials.gov number NCT00093067) and were supplied by Cubist Pharmaceuticals, Inc. (Lexington, MA) (17). Both isolates are *mecA* positive and were baseline bloodstream isolates from patients with documented IE. Previous testing identified isolate B346846 as having the genetic variants *agr* type I, *spa* type 17, and staphylococcal chromosomal cassette *mec* (SCC*mec*) type IV and isolate B341002 as carrying *agr* type II, *spa* type 2, and SCC*mec* type II.

Antimicrobial agents. Vancomycin (lot no. 087K0694), rifampin (lot no. 087K187), and gentamicin (lot no. 016K1120) were purchased from Sigma Chemical Company, St. Louis, MO. Daptomycin (lot no. CDCX01) was obtained from Cubist Pharmaceuticals, Inc. Stock solutions of each antibiotic were freshly prepared at the beginning of each week and were kept frozen at -80°C .

Medium. Mueller-Hinton broth (Becton Dickinson and Co., Sparks, MD) supplemented with calcium and adjusted to physiologic conditions of 50 mg/liter calcium chloride (ionized Ca; 1.03 to 1.23 mmol/liter) and 12.5 mg/liter magnesium was used for all in vitro pharmacodynamic models. Bacto tryptic soy broth (Becton Dickinson and Co.) supplemented with 1% glucose and 50 mg/liter calcium chloride was used to optimize biofilm production in the minimal biofilm eradication concentration (MBEC) assay (38). Calcium quantification was verified in all test broths by the Providence Veterans Affairs Medical Center (Providence, RI) in-house clinical laboratory with an Abbott (Abbott Park, IL) Architect c8000 apparatus. Colony counts were determined by using tryptic soy agar (TSA; Difco, Becton Dickinson).

Susceptibility. The MICs of the study antimicrobial agents were determined by the Etest methodology and broth microdilution according to the guidelines of the Clinical and Laboratory Standards Institute (10, 11). The MICs were also determined at a high inoculum (10^9 CFU/g), as described previously (24), and in the presence of 4 g/dl of human albumin (Rhode Island Blood Bank, Providence), which contained free fatty acids to more closely mimic normal human protein binding (William Craig, University of Wisconsin—Madison, personal communications). The minimum bactericidal concentrations were determined by inspection of the colony counts on wells displaying no visible growth (40). All samples were incubated at 37°C in ambient air for 24 h. Antimicrobial susceptibility was also evaluated in the presence of established 24-h biofilms by using a Calgary biofilm MBEC pin-lid device (9). The MBEC was defined as the minimal concentration of an antibiotic required to eradicate the growth in the biofilm.

In vitro pharmacodynamic infection model with SEVs. A previously described simulated endocardial vegetation (SEV) model was used to evaluate several antibiotic regimens. *S. aureus* was used at a high inoculum to represent the organism density often associated with sequestered infections such as endocarditis (20, 24). A bacterial inoculum of approximately 10^9 CFU/g was achieved in the SEVs by combining 50 μl of a high-inoculum organism suspension, 500 μl of human cryoprecipitate antihemolytic factor from human volunteer donors (Rhode Island Blood Bank), and 5 μl of a platelet-saline suspension (250,000 to 500,000 platelets per clot) in siliconized Eppendorf tubes. After vortex mixing to ensure a homogeneous mixture, a monofilament line and then 50 μl bovine thrombin (5,000 U/ml) were added to each Eppendorf tube. SEVs were then removed from the tubes and inserted into the model. This methodology results in SEVs containing approximately 3 to 3.5 g/dl of albumin and 6.8 to 7.4 g/dl of total protein (20, 28). Recent studies have confirmed that the addition of albumin does not significantly affect the activity of time-kill studies; therefore, additional albumin was not added to the supplemented broth (7, 39).

An in vitro pharmacodynamic model consisting of a 250-ml one-compartment glass apparatus with ports in which the SEVs were suspended was utilized for all simulations (28). The apparatus was prefilled with medium and antibiotics, and over a 72-h period, the medium and antibiotics were administered as boluses into

the central compartment via an injection port. The models were placed in a 37°C water bath throughout the procedure with a magnetic stir bar for thorough mixing of the drug in the model. Fresh media were continuously supplied and removed from the model via a peristaltic pump set (Masterflex; Cole-Parmer Instrument Company, Chicago, IL) to simulate the half-lives of the antibiotics.

Daptomycin was administered to simulate a dose of 6 mg/kg of body weight every 24 h (maximum concentration of drug in plasma [C_{max}], 98.6 $\mu\text{g}/\text{ml}$; half-life, 8 h; area under the concentration-time curve from time zero to 24 h [AUC_{0-24}], 747 $\mu\text{g} \cdot \text{h}/\text{ml}$) (12). Vancomycin was administered to simulate a dose of 1.25 g every 12 h (C_{max} , 45 $\mu\text{g}/\text{ml}$; minimum concentration of drug in plasma [C_{min}], 15 to 20 $\mu\text{g}/\text{ml}$; half-life, 6 h; AUC_{0-24} , 300 $\mu\text{g} \cdot \text{h}/\text{ml}$) (27). Gentamicin was administered to simulate a dose of 1.3 mg/kg every 12 h (C_{max} , 6 $\mu\text{g}/\text{ml}$; C_{min} , 0.4 $\mu\text{g}/\text{ml}$; half-life, 2 h). Rifampin was administered to simulate an intravenous dose of 300 mg every 8 h (C_{max} , 10.5 $\mu\text{g}/\text{ml}$; half-life, 4 h; AUC_{0-24} , 85.7 $\mu\text{g} \cdot \text{h}/\text{ml}$) (30). For the experiments with the combination regimen, the elimination rate was determined for the drug with the shortest half-life; supplemental drug was added for the agent with the longer half-life (5). Experiments with all models were performed in triplicate to ensure reproducibility. In addition, simulations were performed in the absence of antibiotics to ensure the adequate growth of the organisms in the model.

Two SEVs were removed from each model, for a total of six SEVs each at times of 0, 4, 8, 24, 32, 48, and 72 h. Once the SEVs were removed, they were immediately homogenized, diluted, and plated onto TSA; samples were then incubated at 37°C for 24 h, and the colony count was enumerated. Antimicrobial carryover was minimized by serial dilution (10 to 10,000) of plated samples in conjunction with vacuum filtration, as described previously (24). Colonies were counted on filter paper containing a grid with a limit of detection of 10^2 CFU/g.

Activity defined. The reductions in the \log_{10} number of CFU/g over 72 h were determined by plotting time-kill curves. Bactericidal activity (99.9% killing) was defined as a $\geq 3\text{-log}_{10}\text{-CFU/g}$ change and bacteriostatic activity was defined as a $< 3\text{-log}_{10}\text{-CFU/g}$ change in the colony count from that for the initial inoculum at 24, 48, and 72 h. The time required to kill 99.9% of the bacteria present was determined by nonlinear regression (by use of a minimum of four data points) if r^2 was ≥ 0.95 or by visual inspection.

In addition, reductions in the colony counts were determined over a 72-h period and were compared between regimens. Synergy was defined as a $\geq 2\text{-log}_{10}$ decrease in the number of CFU/g between the combination regimen and its most active constituent after 24 h and when the number of surviving organisms in the presence of the combination was $\geq 2\text{ log}_{10}$ CFU/g below the starting inoculum (1). Indifference was defined as a $< 2\text{-log}_{10}\text{-CFU/g}$ increase in killing in comparison to that achieved with the most active single agent. Combinations that resulted in bacterial growth of $> 2\text{ log}_{10}$ CFU/g in comparison to that achieved with the most active single agent were considered antagonistic (1).

Pharmacodynamic analysis. C_{max} -to-MIC ratios, the percentage of time that the concentration remained above the MIC, and AUC_{0-24} -to-MIC ratios were calculated for each antibiotic and were compared to the values provided in the literature. The MICs used in these analyses were obtained from broth microdilution results, since that was the methodology used to obtain the values reported in the literature.

Pharmacokinetic analysis. Samples for pharmacokinetic analysis were obtained through the injection port at 0.5, 1, 2, 4, 8, 24, 32, 48, 56, and 72 h for verification of the target antibiotic concentrations. All samples were stored at -80°C until they were ready for analysis. Daptomycin concentrations were determined by a previously described and validated high-pressure liquid chromatography (HPLC) method (Center for Anti-Infective Research and Development, Hartford, CT) (13). Gentamicin and vancomycin concentrations were determined by a homogeneous particle-enhanced turbidimetric immunoassay (Architect, Multigent; Abbott Diagnostics, Abbott Park, IL) at the Providence Veteran Affairs Medical Center. The gentamicin assay has a range of detection of 0.3 to 10.0 $\mu\text{g}/\text{ml}$ and a between-day sample precision and a coefficient of variation of 1.35% and $\leq 2.75\%$, respectively. The vancomycin assay has a detection range of 0.5 to 80.0 $\mu\text{g}/\text{ml}$ and a between-day sample precision and coefficient of variation of 1.6% and $\leq 5.0\%$, respectively. Rifampin concentrations were evaluated by HPLC (National Jewish Medical and Research Center, Denver, CO), as described previously (30). The half-life, AUC , C_{max} , and C_{min} of the antibiotics were determined by the trapezoidal method with PK Analyst software (version 1.10; MicroMath Scientific Software, Salt Lake City, UT).

Resistance and MIC increases. Changes in the MICs were evaluated for the monotherapy and the combination therapy models at 24, 48, and 72 h. To assess the development of resistance, 100- μl samples were collected at these time points and were plated on TSA plates containing two-, four-, and eightfold the MIC of the respective antibiotic. In addition, MIC testing was conducted for rifampin, daptomycin, vancomycin, and gentamicin by Etest. Samples were evaluated di-

TABLE 1. MIC results obtained with standard and high inocula for clinical isolates and MBEC results for all isolates

| Antimicrobial ^a | MIC ($\mu\text{g/ml}$) ^b | | | | MBEC (mg/liter) ^c | |
|----------------------------|---------------------------------------|---------------|-------------------|---------------|------------------------------|--------------|
| | MRSA B341002 | | MRSA B346846 | | MRSA B341002 | MRSA B346846 |
| | Standard inoculum | High inoculum | Standard inoculum | High inoculum | | |
| Daptomycin | 0.5 | 8 | 0.5 | 8 | 16 | 8 |
| Daptomycin with albumin | 4 | 8 | 4 | 8 | NA ^d | NA |
| Vancomycin | 0.5 | 2 | 1 | 4 | 128 | 64 |
| Vancomycin with albumin | 1 | 4 | 1 | 4 | NA | NA |
| Gentamicin | 0.5 | 0.5 | 0.25 | 0.5 | 256 | 256 |
| Gentamicin with albumin | 0.5 | 0.25 | 0.125 | 0.25 | NA | NA |
| Rifampin | <0.02 | 0.25 | 0.06 | 0.5 | 0.0625 | 0.0625 |
| Rifampin with albumin | <0.06 | <0.06 | <0.06 | <0.06 | NA | NA |

^a For antimicrobials with albumin, albumin was added to the broth at 4 g/dl.

^b The standard inoculum was 5×10^5 CFU/ml, and the high inoculum was 5×10^9 CFU/ml.

^c A Calgary biofilm device was used to determine the antimicrobial activity in a formed biofilm. The values were obtained by determination of the plate counts, and the limit of detection was 2.4 CFU/ml.

^d NA, not applicable.

rectly from the model to prevent the passing of bacteria on antibiotic-containing plates and to optimize the detection of MIC changes. The plates were examined for growth after 24 and 48 h of incubation at 37°C.

Statistical analysis. Changes in bacterial growth (CFU/g) at 4, 8, 24, 48, and 72 h and the time to 99.9% killing were compared by two-way analysis of variance with Tukey's post-hoc test. A *P* value of ≤ 0.05 was considered significant. All statistical analyses were performed with SPSS statistical software (release 15; SPSS, Inc., Chicago, IL).

RESULTS

Susceptibility testing. Daptomycin, vancomycin, gentamicin, and rifampin were active against both of the biofilm-forming MRSA clinical isolates evaluated in this study (Table 1). In the presence of human albumin (4 g/dl), the MICs for daptomycin and vancomycin increased eight- and twofold, respectively. In the presence of high inocula, there were also increases in the daptomycin and vancomycin MICs of 16- and 4-fold, respectively. These results are consistent with those presented in other reports (24) and may be explained by the relatively high and moderate levels of protein binding exhibited by daptomycin (93%) and vancomycin (55%), respectively (15, 27). There was a minimal increase in the gentamicin and rifampin MICs (less than or equal to twofold each) when the MRSA isolates were evaluated in the presence of albumin and/or at high inocula. This is also consistent with the findings presented in previously published studies (24).

In vitro pharmacokinetics and pharmacodynamics. The pharmacokinetic parameters of the antimicrobial agents were within the targeted range and can be found in Table 2. All C_{max} s obtained were within 10% of the targeted C_{max} s and the

vancomycin AUC/MIC ratios ranged from 641 to 1,282 for each clinical isolate.

Biofilm production quantified. Both clinical MRSA isolates exhibited biofilm formation. The biofilm-forming *S. aureus* control strain (strain ATCC 35556) demonstrated biofilm formation with an optical density at 570 nm (OD_{570}) of 0.68 ± 0.08 . The clinical isolates had consistent biofilm production (OD_{570} , 0.65 ± 0.09), and the non-biofilm-forming control isolate did not produce a biofilm (OD_{570} , 0.36 ± 0.04).

Antimicrobial activity in a formed biofilm assay. The daptomycin and vancomycin MBECs were 8 to 16 and 64 to 128 $\mu\text{g/ml}$, respectively, for the clinical isolates. The gentamicin and rifampin MBECs were 256 to 512 $\mu\text{g/ml}$ and 0.0625 $\mu\text{g/ml}$, respectively, for the biofilm-forming clinical isolates, thus indicating that rifampin demonstrates activity in a preformed biofilm assay.

The antimicrobial activities of daptomycin and vancomycin were evaluated alone and in combination with gentamicin or rifampin against a high inoculum (10^9 CFU/g) of biofilm-forming MRSA isolates in a simulated IE vegetation model (Table 3; Fig. 1). Daptomycin monotherapy achieved bactericidal activity against strain B341002 at 8 h and against strain B346846 at 24 h. At 24 h, daptomycin monotherapy demonstrated significantly better activity than daptomycin in combination with rifampin or gentamicin against MRSA B346846 (mean differences, 2.18 [$P = 0.03$] and 4.61 [$P = 0.001$], respectively) and daptomycin plus gentamicin against MRSA B341002 (mean difference, 2.70 [$P = 0.002$]). The mean differences at 24 h also met the criteria for antagonism; rifampin antagonized dapto-

TABLE 2. Targeted values and values of pharmacokinetic parameters obtained with SEV infection model

| Regimen ^a | C_{max} ($\mu\text{g/ml}$) | | Half-life (h) | | AUC_{0-24} ($\mu\text{g h/ml}$) | |
|----------------------------------|---------------------------------------|-----------------------|---------------|-----------------|--|----------|
| | Targeted | Obtained ^b | Targeted | Obtained | Targeted | Obtained |
| Daptomycin, 6 mg/kg every 24 h | 98.6 | 88.57 ± 0.35 | 8 | 7.16 ± 0.59 | 747 | 825 |
| Vancomycin, 15 mg/kg every 12 h | 45 | 42.32 ± 11.9 | 6 | 6.04 ± 0.17 | 300 | 341 |
| Gentamicin, 1.3 mg/kg every 12 h | 6 | 5.70 ± 0.98 | 2 | 2.91 ± 0.87 | | |
| Rifampin, 300 mg every 8 h | 10.5 | 9.67 ± 0.27 | 4 | 5.24 ± 0.29 | 86 | 133 |

^a The regimens are based on those for a 75-kg patient.

^b The values obtained are means \pm standard deviations.

TABLE 3. Change in inoculum from starting inoculum of 5×10^9 CFU/g at 8, 24, and 72 h obtained with SEV infection model

| Antimicrobial(s) | Mean change in bacterial density (log ₁₀ CFU/g) | | | | | |
|-------------------------|--|----------------------|----------------------|--------------|----------------------|--------------------|
| | MRSA B341002 | | | MRSA B346846 | | |
| | 8 h | 24 h | 72 h | 8 h | 24 h | 72 h |
| Growth control | −0.42 | −0.37 | +1.50 ^c | +1.47 | +1.81 | +1.51 |
| Daptomycin | −3.05 ^a | −6.70 ^a | −6.70 ^a | −1.68 | −6.61 ^a | −6.36 ^a |
| Daptomycin + gentamicin | −2.07 ^a | −3.85 ^{a,b} | −6.53 ^a | −0.99 | −1.97 ^{a,b} | −6.61 ^a |
| Daptomycin + rifampin | −3.18 ^a | −5.64 ^a | −6.83 ^a | −0.88 | −3.95 ^{a,b} | −6.19 ^a |
| Vancomycin | −0.11 | −0.70 | −1.40 ^a | −0.13 | −1.11 | −2.04 ^a |
| Vancomycin + gentamicin | −0.56 | −1.40 | −3.17 ^{a,b} | −0.73 | −1.08 | −1.58 ^a |
| Vancomycin + rifampin | −0.06 | −0.73 | −2.58 ^a | −0.22 | −1.16 | −1.81 ^a |

^a $P \leq 0.046$ versus the results for the growth control.
^b $P \leq 0.03$ versus the results for monotherapy.
^c Positive values indicate growth.

mycin’s activity against MRSA B346846, and gentamicin antagonized daptomycin’s activity against both isolates. There were no significant differences in activity between daptomycin alone and daptomycin plus rifampin or gentamicin at 48 or 72 h, nor was synergy or antagonism noted by 48 or 72 h.

Vancomycin monotherapy did not achieve bactericidal activity against either clinical isolate tested at any time point. There was also no significant difference between vancomycin monotherapy and the regimens of vancomycin plus rifampin or vancomycin plus gentamicin at any time point during the 72-h experiment. At 24, 48, and 72 h, the daptomycin-containing regimens had significantly ($P = <0.05$) more activity (as measured by a decline in the mean numbers of CFU/g) than any of the vancomycin-containing regimens (Table 3). Gentamicin and rifampin monotherapies did not demonstrate any significant activity during the study. Resistance (defined as an MIC of $>32 \mu\text{g/ml}$) occurred in the rifampin monotherapy models by 24 h, and the gentamicin MICs increased fourfold within 24 h. The vancomycin and daptomycin MICs varied at each time point but never exceeded $2 \mu\text{g/ml}$.

DISCUSSION

Left-sided IE is a sequestered infection that often yields a high bacterial density (10^8 to 10^{10} organisms per gram of tissue) (4). IE can develop when an organism attaches to the heart valve and forms a vegetation. The limited blood supply to this area and the high bacterial load result in a blunted immune response and limited antimicrobial drug access. As the infection progresses, the rates of bacterial metabolism and cell division are reduced and biofilms may develop as a result of nutrient limitation and autolysin production. Clinical cure can be achieved, but the prolonged administration of bactericidal cell-wall-active antibacterial agents is required to sterilize the vegetation. Treatment success depends on multiple factors, including patient comorbidities, the location of the vegetation (right-sided versus left-sided endocarditis), and surgical intervention (17, 18). Limited antibiotic penetration into the vegetation partially explains why left-sided IE is considered a disease which must be treated surgically. The exact cause of most treatment failures is unknown; however, it is likely related to the ability of the bacteria to form biofilms (14, 19). The bacteria embedded in a biofilm are less susceptible to antibiotics

by virtue of their reduced growth rates, nutrient limitation, and adaptive stress responses (6, 42).

We report here the findings of studies of daptomycin and vancomycin monotherapies and combination therapy with rifampin or gentamicin in an in vitro model of endocarditis caused by biofilm-forming clinical isolates. The addition of gentamicin or rifampin did not significantly improve the activity of daptomycin or vancomycin (Fig. 1). Daptomycin had significantly better activity than vancomycin against both of the biofilm-forming MRSA isolates, and no MIC shifts were observed. Of interest, during the first 24 h, rifampin antagonized and delayed the bactericidal activity of daptomycin against MRSA B346846, and gentamicin antagonized and delayed the bactericidal activity of daptomycin against both isolates. Although contradictory results can often be found in the literature (31), antagonistic activity is often observed when rifampin is added to bactericidal agents in high-inoculum infections and against biofilm-forming *S. aureus* (29, 31, 34). In addition, the inhibition of bacterial RNA synthesis may be responsible for delaying the killing activities of cell-wall-active agents. Clinical studies have rarely demonstrated bactericidal activity with rifampin combination therapy for the treatment of SAIE (25, 31).

In this study, antagonistic or delayed bactericidal activity was observed during the first 24 h when gentamicin was added to daptomycin. This has been observed in clinical studies (16) and in vitro studies (34) with vancomycin, but to our knowledge, this has not yet been observed with daptomycin. We believe that this effect may be isolate dependent and may be due to the biofilm.

The biofilm formation by two MRSA isolates obtained from patients with SAIE was quantified. Both clinical isolates produced 38 to 49% more biofilm than a characterized non-biofilm-forming control (strain ATCC 12228) and slightly less biofilm (5 to 12%) than a characterized biofilm-forming isolate (strain ATCC 35556). The daptomycin MBECs in a 24-h mature biofilm of the clinical isolates were 8 to $16 \mu\text{g/ml}$, which are 4 to 5 serial dilutions higher than the MIC for planktonic bacteria; these concentrations are clinically achievable with a 6-mg/kg dose (C_{max} , $98 \mu\text{g/ml}$). For vancomycin, the MBECs were 64 to $128 \mu\text{g/ml}$, which is 6 to 8 serial dilutions higher than the MICs for planktonic bacteria; these concentrations are not clinically achievable by the use of traditional doses. The rifampin and gentamicin MBECs were 0.062 and $256 \mu\text{g/ml}$,

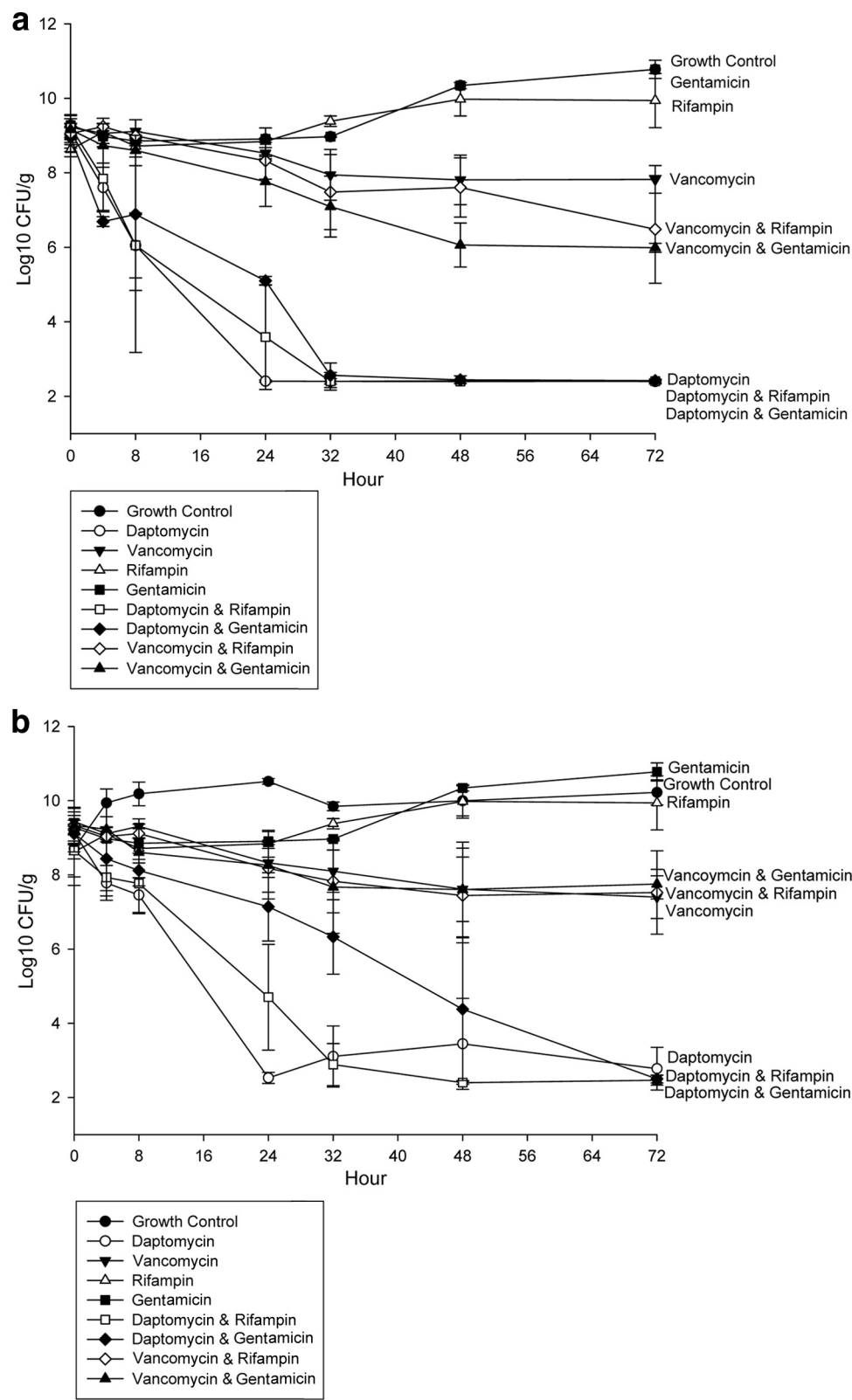


FIG. 1. Activities of antimicrobials tested alone and in combination against MRSA B341002 (a) and B346846 (b). Dapto, daptomycin; Vanco, vancomycin; Gent, gentamicin; Rif, rifampin.

respectively. The observed increase in the MBECs relative to the MICs is consistent with previous reports of antimicrobial resistance when bacteria transition from the planktonic form to the biofilm form (9).

Overall, the daptomycin and vancomycin MICs for both clinical isolates were higher when they were grown in a high inoculum or in the presence of 4 g/dl of albumin. The gentamicin and rifampin MICs were minimally affected by the high inoculum or albumin, consistent with the findings of other published studies (24, 34). Daptomycin and vancomycin exhibited high to moderate levels of protein binding; thus, albumin and a high bacterial inoculum (such as in a biofilm or vegetation) decrease the free drug concentration, which reduces the AUC/MIC ratio and decreases the antimicrobial activity. In the presence of a high inoculum and albumin, the increase in the daptomycin and vancomycin MICs and the preservation of the rifampin and gentamicin MICs were not correlated with activity in the *in vitro* model.

In conclusion, both of the clinical MRSA isolates obtained from patients with IE quantitatively produced biofilms. The addition of gentamicin or rifampin to either vancomycin or daptomycin did not increase their antibacterial activities in a sequestered high-inoculum model of biofilm-forming MRSA IE, and rifampin and gentamicin were shown to delay the bactericidal activity of daptomycin during the first 24 h. Overall, daptomycin monotherapy had significantly better activity against both of the biofilm-forming MRSA isolates than vancomycin.

A limitation of this study is the use of only two clinical MRSA isolates. In addition, we cannot conclude that our results will hold true with treatment durations longer than 72 h. Our findings on the activities of daptomycin and vancomycin monotherapies are consistent with those obtained with clinical, *in vitro*, and animal models published previously; however, until now, biofilm formation has not been quantified (16, 25, 33, 34). To our knowledge, this is the first study to evaluate the activities of these agents against biofilm-forming MRSA in a sequestered high-inoculum model of IE. The results support the use of daptomycin monotherapy for the treatment of biofilm-forming MRSA in a simulated endocarditis vegetation. Nonetheless, our results should be applied to clinical practice with caution. Confirmation of these results in clinical studies is needed before these regimens can be adopted for use for the care of patients.

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